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COMMUNICATION

The Effect of Lipid A Analog E5531 on Membrane Properties of Dipalmitoylphosphatidylcholine

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ABSTRACT

The effect of the lipid A analog E5531 on the phospholipid membrane was determined and compared with that of the lipid A from Escherichia coli *(EC). E5531 decreased the phase transition temperature of dipalmitoylphosphatidylcholine (DPPC) membrane and increased the fluidity and permeability. On the other hand, EC increased the phase transition temperature and decreased the membrane fluidity and permeability. These results suggest that the reason for the difference of biological effects of E5531 and lipid A from EC would be caused by the differences from the effect on the cell membranes*.

Key Words: *Dipalmitoylphosphatidylcholine; Lipid A analog; Membrane fluidity; Permeability.*

INTRODUCTION

Lipid A is a component of bacterial lipopolysaccharides (LPSs), which are present on the major amphiphilic constituents of the leaflet of gram-negative bacteria. This is a potent biological active site (1,2) and induces the prostaglandins, cytokines such as interferon (3), interleukin (4), and tumor necrosis factor (TNF) (5) in mammalian cells such as macrophages and lymphocytes. This compound also induces undesirable toxic effects such as fever and the Schwartzmann bleeding reactions (6,7).

Recently, researchers have focused on the effect of lipid A on the structural and dynamic properties of membranes and revealed that most biological effects induced by lipid A are initiated by binding to a specific receptor (8,9) or by nonspecific intercalation into the lipid matrix of the cell membrane (10). The interaction and subsequent intercalation into the membrane is dependent on the fluidity of the hydrophobic region and/or the supramolecular structure of LPS and lipid A (11). Liu et al. (12) have also reported that lipid A from *Salmonella minnesota* decreased the membrane fluidity and raised

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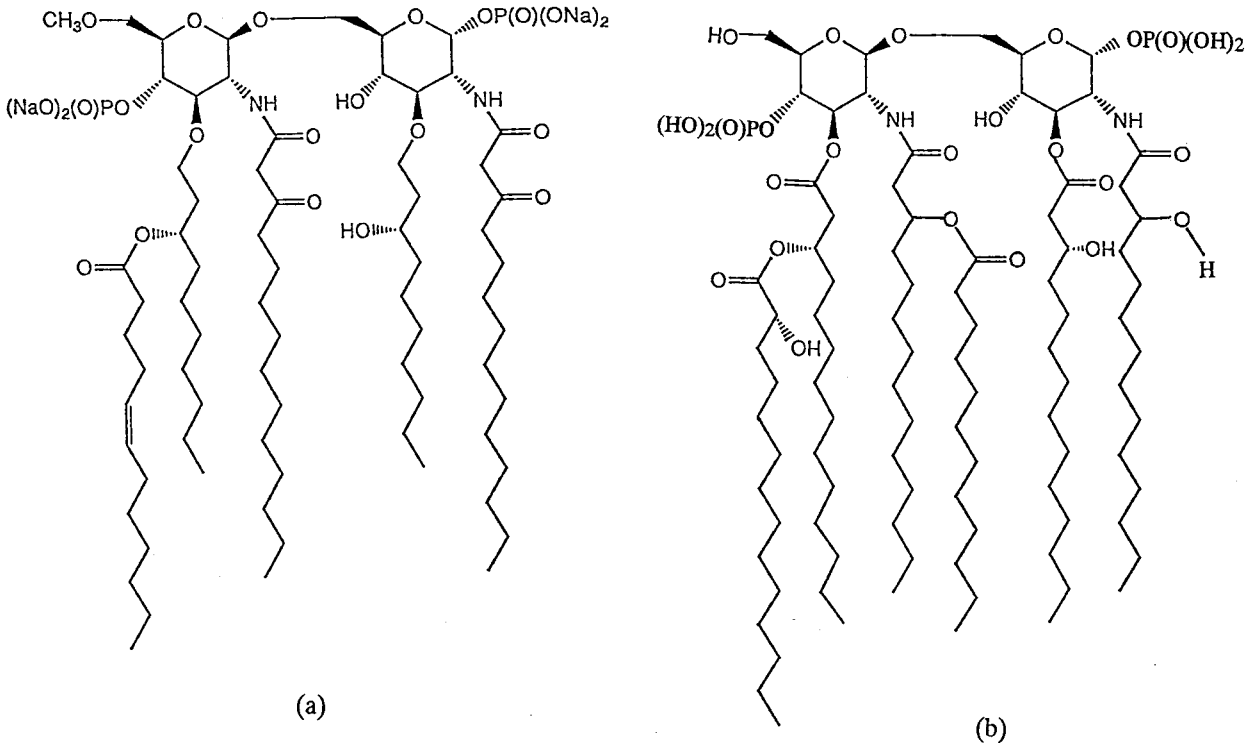

Figure 1. (a) Chemical structure of the synthetic lipid A analog E5531; (b) chemical structure of the lipid A from *Escherichia coli*.

the phase transition temperature of phospholipid membranes. Benedetto et al. (13) suggested that some of the effects produced by lipid A are mediated by a specific molecular reaction at the cell surface membrane, and that the physicochemical properties of the membrane may be important determinants of the biological activity of lipid A.

Researchers have also focused recently on the synthesis of lipid A analogs with low toxicity. The synthetic disaccharide lipid A analog E5531 (Fig. 1a) has low toxicity and retains various useful biological activities (e.g., reduction of TNF production) possessed by lipid A (14). This compound has been found to be a specific LPS antagonist in an LPS-binding assay, and it inhibits induced TNF production in monocytes/macrophages induced by lipid A and LPS (15). Its anticipated use is as a drug for the treatment of septic shock (16,17).

The studies mentioned above for verification of the useful effects of E5531 were mainly conducted by comparing them with the toxic effects of the lipid A and LPS from *Escherichia coli* (EC). In this study, we compared the effect of E5531 and the lipid A from EC (Fig. 1b) on dipalmitoylphosphatidylcholine (DPPC) as a model membrane of cell surfaces; we used several physicochemical techniques, such as dynamic light scattering (DLS) and fluorescence spectroscopy. In addition, we investigated the correlation of these effects with the initiation of biological activities.

EXPERIMENTAL

Materials

E5531 was obtained from Eisai Chemical Company, Limited (Ibaraki, Japan). Lipid A from *Escherichia coli* F583 (Rd mutant) was purchased from Sigma Chemical Company, Limited (St. Louis, MO). L-α-Dipalmitoylphosphatidylcholine (DPPC) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Wako Pure Chemical Industrial Limited (Osaka, Japan). Calcein (3,3'-bis [*N*,*N*-bis (carboxymethyl) aminomethyl]-fluorescein) was supplied by Dojin Company, Limited (Kumamoto, Japan). Lactose hydrous, sodium phosphate monobasic, sodium phosphate dibasic, and sodium hydroxide were purchased from Mallinckrodt Company, Limited (Paris, KY).

Methods

Preparation of the Aggregates from the Lipid Mixtures

The aggregates from E5531/DPPC and the lipid A from EC/DPPC were prepared by the method of Dijkstra et al. (18). DPPC was dissolved in chloroform, and E5531 or EC was dissolved in methanol. These stock solutions were then mixed at a suitable ratio. The solvents were evaporated under a steam of nitrogen gas at 70°C. The lipid film was hydrated to give a total concentration of the total lipids of 1 mM with 4.25 mM phosphate-NaOH buffer containing 10% lactose (pH 7.3). The lipid dispersion was then sonicated with a probe-type sonicator (Tomy Seiko Co., Ltd., Tokyo, Japan) at 50°C for 10 min.

Determination of the Size of the Lipid Aggregates

The size of the aggregates in the lipid mixtures was determined at 25°C by the DLS techniques using a laser particle analyzer (model DLS-7000DL, Ohtsuka Electronics Co., Ltd., Osaka, Japan). The data were analyzed by the histogram method (19), and the weight-averaged aggregate sizes were evaluated.

Determination of the Phase Transition Temperatures of the Lipid Mixtures

The membrane fluidity of the E5531/DPPC and EC/DPPC aggregates was determined using a fluorescence polarization technique (DPH probe) reported by Iwamoto et al. (20). DPH was added at 1 mol% of total lipids. All fluorescence measurements were carried out using a model F-4500 fluorescence spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan) equipped with a thermoregulated cell compartment (Atago Coolnics model REX-C10, Atago Co., Ltd., Tokyo, Japan). The degree of polarization P was calculated using the following equation:

$$P = (I_{VV} - C_f \cdot I_{VH})/(I_{VV} + C_f \cdot I_{VH})$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($= I_{HV}/I_{HH}$) is the grating correction factor.

Effect of E5531 and EC on the Permeability of Dipalmitoylphosphatidylcholine Membrane

The effect of E5531 and EC on the permeability of DPPC membrane was determined using fluorescence techniques (21). The lipid mixtures were dispersed in 2.5 ml of 70 mM calcein solution (pH 7.3) with sonication at 50°C for 60 min and then cooling to 25°C. The total lipid concentration was 2 mM.

The untrapped calcein was eluted from Sephadex G-50 gel with the void fraction with 4.25 mM phosphate-NaOH, 10% lactose buffer solution (pH 7.3), and 0.5 ml of the fraction was added to 2.5 ml of rat plasma. The permeability of the E5531 aggregates was evaluated fluorometrically by monitoring the leakage of calcein during incubation with rat plasma at 37°C. The percent leakage of calcein was calculated according to the following equation:

$$\text{Leakage (\%)} = [(F - F_0)/(F_8 - F_0)] \times 100$$

where F_0 represents the initial fluorescence intensity at time zero, F is the fluorescence intensity monitored during the incubation at 37°C, and F_8 denotes the maximum fluorescence intensity after lysis of the aggregates by the addition of 0.1 ml of 10% Triton X-100.

RESULTS

Effect of E5531 and *Escherichia coli* on the Size of Dipalmitoylphosphatidylcholine Liposomes

Table 1 shows the weight-averaged size of the aggregates of the E5531/DPPC and EC/DPPC mixtures evalu-

Table 1

Size of Aggregates for the Mixtures of DPPC/E5531 and from DPPC and the Lipid A from Escherichia coli

	Mole Fraction of Lipids in the Mixtures			
	0	0.1	0.3	0.5
Size of aggregates (nm)				
E5531	18.9 ± 7.9	20.0 ± 7.3	17.4 ± 6.5	19.4 ± 7.9
Escherichia coli	18.9 ± 7.9	24.8 ± 5.2	25.2 ± 3.8	24.3 ± 4.0

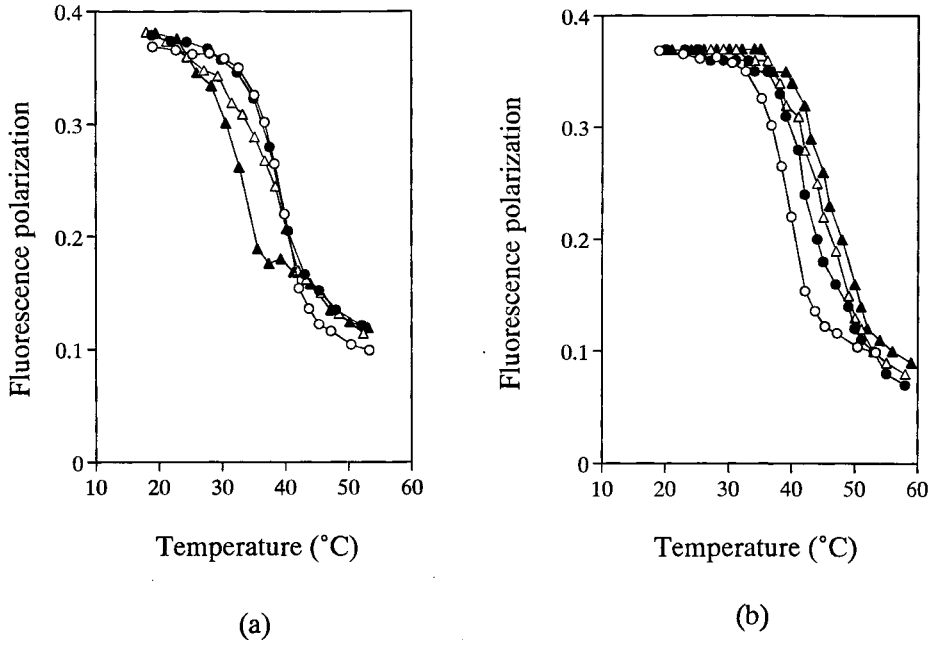

Figure 2. Relationship between incubation temperature and fluorescence polarization (DPH probe) as a function of the lipid mole fraction in the lipid mixture: (a) E5531/DPPC mixtures with ○, $X_{E5531} = 0$; ●, $X_{E5531} = 0.1$; △, $X_{E5531} = 0.3$; ▲, $X_{E5531} = 0.5$; (b) *E. coli*/DPPC mixtures with ○, $X_{EC} = 0$; ●, $X_{EC} = 0.1$; △, $X_{EC} = 0.3$; ▲, $X_{EC} = 0.5$.

ated by DLS measurements at different E5531 mole fraction (X_{E5531}) and EC mole fraction (X_{EC}) ranges. The mean diameters for E5531/DPPC and EC/DPPC mixtures were almost 20 and 25 nm, respectively, and independent of X_{E5531} and X_{EC}.

Effect of the E5531 and *Escherichia coli* on the Fluidity of Dipalmitoylphosphatidylcholine Membrane

The membrane fluidity of the E5531/DPPC and EC/DPPC mixtures was evaluated using fluorescence polarization techniques (Fig. 2). The fluorescence polarization of DPH in DPPC liposomes decreased markedly around 40°C, indicating that the phase transition of the DPPC bilayer from gel to liquid crystal state occurs at this temperature. This result is in good agreement with the reported value (22). The phase transition of the lipid mixtures was dependent on X_{E5531}. At $X_{E5531} = 0.1$, 0.3, and 0.5, the phase transition temperatures were 38°C, 37°C, and 33°C, respectively. As X_{E5531} increased, the fluorescence polarization decreased. These results indicate that, with the increase in X_{E5531}, a more fluid membrane was formed, and the cooperative interaction between the DPPC molecules decreased. On the other hand, at $X_{EC} =$ 0.1, 0.3, and 0.5, the phase transition temperatures were 44°C, 46°C, and 47°C, respectively. These results indicate that, with the increase in X_{EC}, more rigid membranes were formed, and the cooperative interaction between the DPPC molecules increased.

Effect of the E5531 and *Escherichia coli* on the Permeability of Dipalmitoylphosphatidylcholine Membrane

The effect of E5531 and EC on the permeability of the DPPC membrane was evaluated on the basis of the leakage profile of calcein at 37°C. Figure 3 shows the leakage of calcein from E5531/DPPC and EC/DPPC aggregates (pH 7.3), indicating that the addition of E5531 (20 mol%) increased the permeability of DPPC membrane; on the other hand, the addition of EC (20 mol%) decreased the permeability of the membrane.

DISCUSSION

In this study, we concluded that E5531 decreased the phase transition temperature of DPPC membrane and increased the fluidity and permeability. On the other

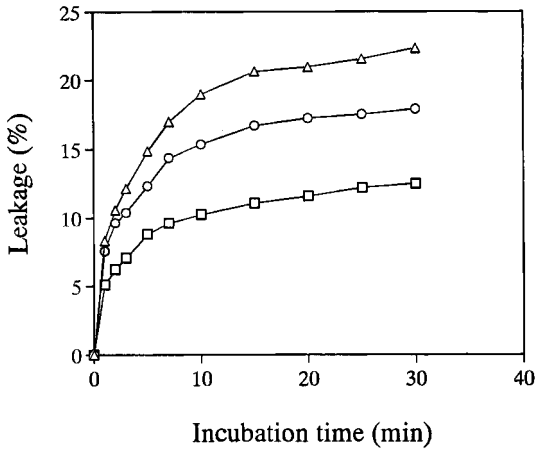

Figure 3. Relationship between incubation time of the lipid mixtures and the leakage of calcein at 37°C: ○, DPPC liposome; △, E5531/DPPC mixtures ($X_{E5531} = 0.2$); □, *E. coli*/DPPC mixtures ($X_{EC} = 0.2$).

hand, the addition of EC increased the phase transition temperature and decreased the membrane fluidity and permeability. These differences may be correlated with the initiation of biological effects induced by these lipids.

For the mechanism of initiation of various biological responses during endotoxin shock induced by lipid A, it has been proposed that lipid A triggers its noxious effects by acting specifically on a receptor site (23) or nonspecifically through membrane lipids (13,24), while others have observed specific binding proteins in the serum or at the cell surfaces (8,9,22,25). For example, the 60-kDa glycoprotein LBP (lipopolysaccharide binding protein) was found to complex with LPS by binding to the lipid A portion of LPS independently of the saccharide moiety (22). The resulting LPS-LBP complex is then recognized by a membrane-bound cell surface ''receptor'' protein called CD14, which finally triggers the biological response (9). In any case, it seems important to note that, after binding of LPS to the membrane, whether specific or unspecified, in a second step the decisive signal that causes the biological response must be given, probably by a membrane protein. For the signal translation, the formation of a nonlamellar inverted structure of lipid A leading to disturbances at or around the ''binding places'' at the cell surface due to the concave curvature of the lipid A bilayer regions might be a prerequisite.

Based on our results, it will be assumed that the intercalation of the E5531 increased the fluidity of that region of the cell membranes and that the biological action of E5531 and molecular mechanism of E5531 interaction with cell membranes will be different from those of EC. It has been reported that E5531 is a lipid A antagonist, that it will bind the LPS receptor, and that the affinity of E5531 to bind the receptor is larger than that for lipid A (14,17). Kawata et al. (15) also reported that E5531 blocked the induction of TNF-α by lipid A and LPS from EC and reduced LPS-induced lethality in mice (15). In addition, Kobayashi et al. (16) suggested that E5531 suppressed the hepatic injury in mice induced by LPS from EC in an apparently competitive manner. Based on the results from our study and these reports, it will be assumed that these useful effects of E5531 will be obtained not only from the difference from the affinity to the LPS receptor, but also from the difference from the interaction with the cell membrane. In other words, for initiation of the useful biological effects, E5531 will bind specifically on a receptor site and will intercalate nonspecifically through membrane lipids and increase the membrane fluidity.

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